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Role for the outer membrane ferric siderophore receptor PupB in signal transduction across the bacterial cell envelope

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The outer membrane protein PupB of *Pseudomonas putida* WCS358 facilitates transport of iron complexed to the siderophores pseudobactin BN8 and pseudobactin BN7 into the cell. Its synthesis is induced by the presence of these specific siderophores under iron limitation. The signal transduction pathway regulating siderophore-dependent expression of *pupB* was shown to consist of two regulatory proteins, PupI and PupR, and the PupB receptor itself. Mutational analysis of the regulatory genes suggested that PupI acts as a positive regulator of *pupB* transcription, whereas PupR modifies PupI activity dependent on the presence of pseudobactin BN8. PupI and PupR do not share homology with the classical bacterial two-component systems but display significant similarity to the FecI and FecR proteins of *Escherichia coli* involved in regulation of ferric dicitrate transport. The function of the PupB receptor in *pupB* regulation was studied by the use of chimeric receptor proteins composed of PupB and the ferric pseudobactin 358 receptor PupA. This experiment revealed that PupB is involved in the initiation of the signal transduction pathway, implying a so far unique role for an outer membrane protein in signal transduction.

Key words: outer membrane receptor/*Pseudomonas putida*/siderophores/signal transduction

Introduction

Iron is essential for the growth of most microorganisms, but its biological availability in the environment is often restricted. Bacteria respond to iron deprivation by producing small water-soluble iron binding compounds, called siderophores, which deliver iron to the cell via specific high-affinity transport systems (Neilands, 1981, 1982). Plant-growth promoting *Pseudomonas putida* WCS358 synthesizes under iron limitation the fluorescent siderophore pseudobactin 358, which is structurally related to the siderophores produced by other fluorescent pseudomonads (Geels and Schippers, 1983; van der Hofstad *et al.*, 1986). This group of siderophores, called pseudobactins or pyoverdines, are composed of a fluorescent chromophore attached to a peptide moiety, which differs in length and composition between siderophores produced by different *Pseudomonas* strains. *P.putida* WCS358 has the capacity to exploit a large number of pseudobactins of heterologous origin for iron acquisition (Bakker *et al.*, 1990).

The ability of a fluorescent *Pseudomonas* strain to utilize a given pseudobactin is related to the presence of an outer membrane receptor which is specific for that ferric pseudobactin complex and facilitates its uptake into the cell. In addition, less specific proteins are involved in transport of ferric pseudobactins across the inner membrane and the release of iron (Marugg *et al.*, 1989; Koster *et al.*, 1993). In *P.putida* WCS358, two ferric pseudobactin receptors, PupA and PupB, have been characterized. The PupA receptor is involved in iron transport via the native siderophore pseudobactin 358 (Bitter *et al.*, 1991), whereas PupB functions in transport of two heterologous siderophores, namely pseudobactins BN8 and BN7 (Koster *et al.*, 1993). The two receptor proteins share considerable sequence homology and are both functionally dependent on the inner membrane proteins TonB, ExbB and ExbD (Bitter *et al.*, 1993), which provide energy required for the transport process (for a review, see Postle, 1990). Mutants of strain WCS358 deficient in PupA or PupB production retain the ability to utilize pseudobactin 358 or pseudobactin BN8, respectively, albeit with reduced efficiency (Bitter *et al.*, 1991; Koster *et al.*, 1993). This implies the presence of additional receptors for these ferric siderophores in this strain.

Synthesis of PupA and pseudobactin 358 is regulated by the concentration of available iron (Marugg *et al.*, 1988). PupB expression is also iron-controlled but has in addition an absolute requirement for the presence of one of its cognate pseudobactins (Koster *et al.*, 1993). A similar type of regulation has been described for the enterobactin- and ferrioxiamine B receptor of *Pseudomonas aeruginosa* (Cornelis *et al.*, 1987; Poole *et al.*, 1990) and for the ferric dicitrate transport system of *Escherichia coli* (Hussein *et al.*, 1981; Pressler *et al.*, 1988). In addition to pseudobactins BN8 and BN7, other heterologous pseudobactins also induce the synthesis of specific outer membrane proteins in *P.putida* WCS358 which suggests the presence of multiple inducible receptors for ferric pseudobactins in this strain. The large variety of ferric siderophore uptake systems reflects the importance of iron competition in the natural habitat of this bacterium.

Siderophore-dependent regulation implies the presence of a signalling system capable of monitoring a specific siderophore and converting this signal into a cellular response. Induction of synthesis of the *E.coli* ferric dicitrate transport system is mediated by two proteins, FecI and FecR, which do not show homology to other known bacterial regulatory proteins (van Hove *et al.*, 1990). Their genes are located immediately upstream of the *fecA* gene encoding the ferric dicitrate outer membrane receptor. It has been proposed that FecI functions as a transcriptional activator of the *fec* genes and FecR as a sensor, repressing FecI

activity in the absence of ferric dicitrate (van Hove *et al.*, 1990). Expression of the ferric enterobactin receptor PfeA in *P.aeruginosa* is under control of two regulatory proteins, PfeR and PfeS (Dean and Poole, 1993), which display homology to the histidine kinase sensors and response regulators of a large family of bacterial two-component regulatory systems (Albright *et al.*, 1989). Thus interestingly, two evolutionarily distinct signal transduction systems are employed for siderophore-dependent regulation of the *fec* genes in *E.coli* and the *pfeA* gene in *P.aeruginosa*.

The aim of the present study is to investigate the regulation of PupB expression in *P.putida* WCS358. Two genes are identified upstream of the *pupB* gene, *pupI* and *pupR*, which are involved in the siderophore-dependent regulation of PupB expression. The predicted translation products exhibit high similarity to the FecI and FecR proteins of *E.coli* providing evidence for a conservation of this regulatory system in Gram-negative bacteria. Furthermore, it is demonstrated that the stimulus to which this two-component system responds is not the ferric siderophore complex directly but a signal transduced by the PupB receptor upon transport of its substrate. This is to our knowledge the first example of an outer membrane protein that acts as a component of a signal transduction cascade.

Results

Identification and characterization of pupI and pupR

Previously, a transcriptional unit upstream of the *pupB* gene was shown to be essential for PupB expression (Koster *et al.*, 1993). In order to identify and characterize gene(s) controlling PupB synthesis, the nucleotide sequence of a 1.7 kb region immediately upstream of *pupB* was determined. A physical map encompassing this region is depicted in Figure 1. Two open reading frames (ORFs), designated *pupI* and *pupR*, were found in the same transcriptional orientation as the *pupB* gene (Figure 2). The codon usage of the two ORFs is characteristic of *Pseudomonas* species (Viebrock and Zumft, 1988; Wong and Abdelal, 1990). The stop codon of the *pupI* ORF overlaps with the start codon of *pupR* (Figure 2) suggesting that *pupI* and *pupR* are cotranscribed in one operon. The putative translation initiation sites of *pupI* and *pupR* located at position 113 and 631 are not preceded by obvious Shine–Dalgarno sequences.

The *pupI* ORF potentially encodes a protein of 173 amino acid residues (mol. wt, 19 474) and the putative PupR protein is 324 amino acid residues (mol. wt, 35 846). To identify potential transmembrane helices, hydrophobicity analysis was performed on the deduced PupI and PupR proteins (von Heijne, 1992). No putative transmembrane domains were predicted for PupI which is suggestive of a cytoplasmic location for this protein. PupR contains two potential transmembrane segments between residues 85–105 and 238–258 (Figure 3) and may therefore represent an integral inner membrane protein. The amino acid sequences of PupI and PupR were compared with known proteins present in the SwissProt sequence database. The only significant sequence similarity found was with the FecI and FecR proteins (Figure 3), a two-component system regulating ferric dicitrate transport in *E.coli* (van Hove *et al.*, 1990). The PupI protein shows an overall identity of 42.8% with the FecI protein, and a helix–turn–helix DNA binding motif (Dodd and Egan, 1990) is present in both proteins at

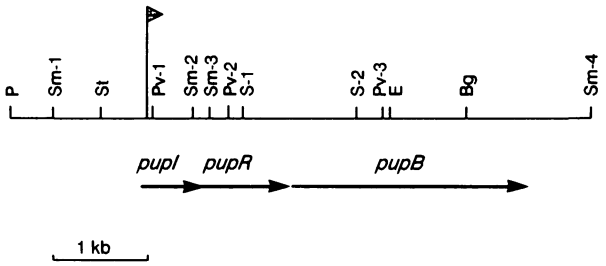

Fig. 1. Physical map of the DNA region containing *pupI*, *pupR* and *pupB*. Indicated are the ORFs and their direction of transcription. The flag represents the position of the Tn*5* insertion abolishing *pupB* expression in strain KV51. Bg, *Bgl*II; E, *Eco*RI; Pv, *Pvu*II; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; St, *Stu*I.

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1     GACCAGTTGCCGTCGCGGCATTTGTCGCGCGCGCGTGTTGCAGGGCCGCCAGGCATGTAT
                                                          pupI
61    ATATGATAAGCATTATCGTTTGCAGTAATGACTCACCTGGCGGCCGCACACCATGCTCCC
                                                          M  L  P
121   TTCCTCTGATCCCCTTTTGTGCGATGTCGCGCTGCTCTACCGCCAGCAGCACAGCTGGTT
        S  S  D  P  L  L  C  D  V  A  L  L  Y  R  Q  Q  H  S  W  L
181   GACGCGCTGGCTCAGGCAGCGCCTGAATTGCTCGCAAAGCGCAGCGGACCTGGCCCAAGA
        T  R  W  L  R  Q  R  L  N  C  S  Q  S  A  A  D  L  A  Q  D
241   CACCTTCATCCGGCTGTTGAACAAGGAGCAGGTGCCGCAACTGCATGCGCCGCGTACCTT
        T  F  I  R  L  L  N  K  E  Q  V  P  Q  L  H  A  P  R  T  F
301   TCTGGCCAAGGTGGCGCAAAGCGTGTTGTGTAACCACTACCGGCGGCAAAAGCTCGAACG
        L  A  K  V  A  Q  S  V  L  C  N  H  Y  R  R  Q  K  L  E  R
361   CGCCTACCTTGAGGCGCTGGCGATGCTGCCTGAGCCGGTGGTGCCAAGCCTGGAGACCCA
        A  Y  L  E  A  L  A  M  L  P  E  P  V  V  P  S  L  E  T  Q
421   GGCGATCCTGCTGGAAACCCTGATCGCCCTCGACGCGGCCCTCGATGGCTTGGAGCGCCC
        A  I  L  L  E  T  L  I  A  L  D  A  A  L  D  G  L  E  R  P
481   GGTGCGCGAGGCGTTCCTGTTGTCGCAGGTGGATGGCCTGGGGCACACCGAAATCGCGCA
        V  R  E  A  F  L  L  S  Q  V  D  G  L  G  H  T  E  I  A  Q
541   ACGGCTGGCCGTGTCGGTCACCACGGTCAAGCGCTACATCATCAAGGCAGGTGCACTGTG
        R  L  A  V  S  V  T  T  V  K  R  Y  I  I  K  A  G  A  L  C
                                                    pupR
601   CATCATGCTCGACCACAGCCTGGACCTGCCATGAACGGCCAGGGCGCAACGTCGATCCCG
        I  M  L  D  H  S  L  D  L  P
                                                M  N  G  Q  G  A  T  S  I  P
661   GGCGAGGTGGCCGAGCAGGCCATGCACTGGCACCTCGAACTGCAAGAGCCGGCCGTCAGT
        G  E  V  A  E  Q  A  M  H  W  H  L  E  L  Q  E  P  A  V  S
721   GCCGCCACCCTGGCCGCCTGCATGAGCTGGCGCCAGGCGCACCCGCTACATGAACATGCC
        A  A  T  L  A  A  C  M  S  W  R  Q  A  H  P  L  H  E  H  A
781   TGGCAACGGACCCAAGTGTTTGCCCAACGCCTGCGCGAAATGCGCAGCCCGGGCCAGCGC
        W  Q  R  T  Q  V  F  A  Q  R  L  R  E  M  R  S  P  G  Q  R
841   CCCTTGGCCCATGCGGCGCTGCGGCCGCAGCAGTCGCGGCGCACTGCGCTCAAGCAATTG
        P  L  A  H  A  A  L  R  P  Q  Q  S  R  R  T  A  L  K  Q  L
901   TCGCTGCTGATGGCCGCAGGCGCAGGGGCCTGGTACTTGAAGGACGCCGCGCTGGTACAA
        S  L  L  M  A  A  G  A  G  A  W  Y  L  K  D  A  A  L  V  Q
961   GACTGGCGCGCTGATTACCACAGCCGCATCGGCGAGCAGCGGCGCCTGACCCTGGCCGAT
        D  W  R  A  D  Y  H  S  R  I  G  E  Q  R  R  L  T  L  A  D
1021  GGCACCCAGGTACAGCTGAACACCGACAGCGCCCTCAATGTGGCATTCGACCAGCAGGCA
        G  T  Q  V  Q  L  N  T  D  S  A  L  N  V  A  F  D  Q  Q  A
1081  CGCCGCCTGAGGCTCGTGCGCGGCGAGATGCTGATCACCCGCCCCGCCCTCGCCGATAGC
        R  R  L  R  L  V  R  G  E  M  L  I  T  R  P  A  L  A  D  S
1141  CGCCCACTGTGGGTAGACACCGAGCACGGCCGCCTTGAGTCGACGCTGGCGCAGTTCAAT
        R  P  L  W  V  D  T  E  H  G  R  L  E  S  T  L  A  Q  F  N
1201  GTGCGGCTGCACGGCCAACACACCCAGGCCACCGTGTATCAGGGCAGCGTGGCGCTGCAA
        V  R  L  H  G  Q  H  T  Q  A  T  V  Y  Q  G  S  V  A  L  Q
1261  CCAGCCCTGCATGCCTACCCGCCCATCCTGCTGGGCGCCGGTGAACAGGCCAGTTTCAAC
        P  A  L  H  A  Y  P  P  I  L  L  G  A  G  E  Q  A  S  F  N
1321  CAGCAGGGCTTGCTGGCGCGGCAGGCCGTGGCCGCTGTCGCCCCCGCCTGGAGCCAAGGC
        Q  Q  G  L  L  A  R  Q  A  V  A  A  V  A  P  A  W  S  Q  G
1381  ATGCTGGTTGCTCAAGGCCAGCCCCTGGCTGCGTTTATCGAAGACCTGGCCCGCTATCGC
        M  L  V  A  Q  G  Q  P  L  A  A  F  I  E  D  L  A  R  Y  R
1441  CGCGGACACCTGGCCTGCGACCCGGCCTTGGCCGGCCTGCGTGTATCCGGCACGTTCCCG
        R  G  H  L  A  C  D  P  A  L  A  G  L  R  V  S  G  T  F  P
1501  CTGGAAAACACCGACAAGATCATTGCTGCCGTGGCAGAAACCTTGCAGTTGGAGGTGCAG
        L  E  N  T  D  K  I  I  A  A  V  A  E  T  L  Q  L  E  V  Q
1561  CATTTCACCCGCTACTGGGTGACATTGAAGCCGCGCATGGCCTGACGCAAAAAAAGTTGC
        H  F  T  R  Y  W  V  T  L  K  P  R  M  A
1621  GCAGGGGTGGTCCGATTTCGCGCCTCACGAGACTTAACCCTCAGCACTTCAAATCAATGG
                                                          pupB
1681  GGGAACACTTGTGAATCACACCGCACGC
                    M  N  H  T  A  R
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Fig. 2. Nucleotide sequence of the DNA fragment carrying *pupI* and *pupR*. The deduced amino acid sequences are presented in one-letter symbols. The putative Fur repressor binding site upstream of *pupI* is underlined.

an equivalent position (Figure 3). The PupR and FecR proteins share 36.6% identity with the highest homology in their C-termini (Figure 3). Only the most N-terminal putative transmembrane domain of PupR is conserved in the FecR protein.

To study the regulation of *pupI* and *pupR* expression, a transcriptional *pupI–lacZ* fusion construct (pMW1) was made. Strain WCS358 harbouring this construct displayed iron-repressed β-galactosidase activity. The activity was ~10-fold higher under iron-depleted than under iron-sufficient conditions and was not increased by the presence of pseudobactin BN8 (Table I). A sequence was identified in the promoter region showing homology to the binding site for the Fur protein of *E.coli* (de Lorenzo *et al.*, 1987) (Figure 2). Fur acts as a repressor of transcription in the presence of sufficient iron, and has been identified in many different bacteria including *P.putida* WCS358 (V.Venturi, personal communication).

Functional analysis of PupI and PupR

The previously described transposon insertions abrogating PupB synthesis, including the chromosomal Tn*5* insertion in mutant KV51 (Koster *et al.*, 1993), were all located in the *pupI* ORF (Figure 1). Mutant KV51, lacking the *pupI* and presumably also the *pupR* gene product, was impaired in the synthesis of detectable amounts of the PupB receptor (Figure 4). A transcriptional *pupB–lacZ* fusion (pMW2) was used to study the transcriptional regulation of the *pupB* gene. β-galactosidase activity in strain WCS358(pMW2) was increased 3-fold in low iron conditions and ~12-fold in response to the presence of pseudobactin BN8 relative to the activity in high iron conditions (Table II). The pseudobactin-dependent induction was completely abolished in the *pupI*::Tn*5* mutant, consistent with a role of this locus in controlling PupB expression at the transcriptional level. The induction could be restored by the introduction of plasmid pMC1 carrying *pupI* and *pupR*. The level of promoter activity in the complemented mutant was increased discernibly relative to the wild-type strain which could be due to the presence of multiple copies of *pupI* and *pupR*. Complementation with plasmid pMC2 carrying only *pupI* resulted in *pupB* expression irrespective of the presence of pseudobactin BN8 (Table II). This suggested a role for PupR in negatively regulating *pupB* expression in the absence of the siderophore.

To assess the function exerted by the PupR protein, a chromosomal *pupR* mutant of strain WCS358 was constructed. For this purpose, the internal *Sma*I fragment of the *pupR* gene on pEW4, a pEMBL18 derivative, was replaced with the Ω interposon containing a streptomycin resistance gene resulting in plasmid pEW5. Since this plasmid cannot replicate in *Pseudomonas*, introduction of pEW5 into strain WCS358 could only result in streptomycin resistant colonies by homologous recombination of the

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PUPI    ML--PSSDPLLCDVALLYRQQHSWLTRWLRQRLNCSQSAA  38
FECI    MSDRATTTASLT-FESLYGTHHGWLKSWLTRKLQSAFDAD  39

PUPI    DLAQDTFIRLLNKEQVPQLHAPRTFLAKVAQSVLCNHYRR  78
FECI    DIAQDTFLRVMVSETLSTIRDPRSFLCTIAKRVMVDLFRR  79

PUPI    QKLERAYLEALAMLPEPVVPSLETQAILLETLIALDAALD  118
FECI    NALEKAYLEMLALMPEGGAPSPEERESQLETLQLLDSMLD  119

PUPI    GLERPVREAFLLSQVDGLGHTEIAQRLAVSVTTVKRYIIK  158
FECI    GLNGKTREAFLLSQLDGLTYSEIAHKLGVSISSVKKYVAK  159

PUPI    AGALCIMLDHSLDLP  173
FECI    AVEHCLLFRLEYGL   173

PUPR    MNGQGATSIPGEVAEQAMHWHLELQEPAVSAATLAACMSW  40
FECR    MNPLLTDS-RRQALRSASHWYAVLSGERVSPQQEARWQQW  39

PUPR    RQAHPLHEHAWQRTQVFAQRLREMRSPGQRPLAHAALR-P  79
FECR    YEQDQDNQWAWQ--QV--ENLRNQLGGVPGDVASRALHDT  75

PUPR    QQSRRTALKQLSLLMAAGAGAWYLKDAALVQDWRADYHSR  119
FECR    RLTRRHVMKGLLLLLGAG-GGWQLWQSETGEGLRADYRTA  114

PUPR    IGEQRRLTLADGTQVQLNTDSALNVAFDQQARRLRLVRGE  159
FECR    KGTVSRQQLEDGSLLTLNTQSAADVRFDAHQRTVRLWYGE  154

PUPR    MLITRPALADSRPLWVDTEHGRLESTLA-QFNVRLHGQHT  198
FECR    IAITTAKDALQRPFRVLTRQGQL-TALGTEFTVRQQDNFT  193

PUPR    QATVYQGSVALQPALHAYPPILLGAGEQASFNQQGLLARQ  238
FECR    QLDVQQHAVEVLLASAPAQKRIVNAGESLQFSASEFGAVK  233

PUPR    AVAAVAPAWSQGMLVAQGQPLAAFIEDLARYRRGHLACDP  278
FECR    PLDDESTSWTKDILSFSDKPLGEVIATLTRYRNGVLRCDP  273

PUPR    ALAGLRVSGTFPLENTDKIIAAVAETLQLEVQHFTRYWVT  318
FECR    AVAGLRLSGTFPLKNTDAILNVIAQTLPVKIQSITRYWIN  313

PUPR    LKPRMAX  325
FECR    ISPL     317
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Fig. 3. Alignment of the deduced protein products of *pupI* and *pupR* with the FecI and FecR proteins of *E.coli*, respectively. Vertical lines indicate identical residues. The conserved helix–turn–helix motif found in PupI and FecI and the putative transmembrane domains in PupR and FecR are underlined.

Table I. Expression of a *pupI–lacZ* fusion in WCS358, KV51 and BWV29 grown under different conditions

		β-Galactosidase activity (U)		
Strain	Genomic mutation	+Fe	−Fe	ps.BN8
WCS358	–	64	700	616
KV51	*pupI*::Tn*5*	12	501	468
BWV29	*pupR*::Ω	43	515	479

All strains harbour plasmid pMW1 carrying the transcriptional *pupI–lacZ* fusion. Cells were grown in iron-deficient RSM medium (−Fe), or in RSM medium supplemented with either 100 μM $FeCl_3$ (+Fe), or 40 μM pseudobactin BN8 (ps.BN8).

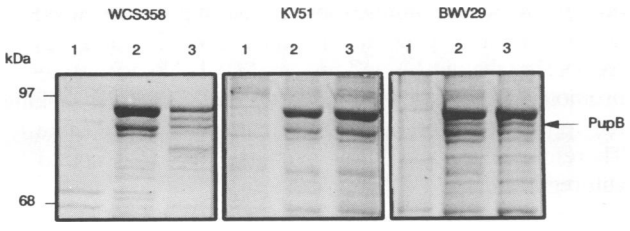

Fig. 4. Expression of the PupB protein in wild-type strain WCS358, *pupI*::Tn*5* mutant KV51, and the *pupR*::Ω mutant BWV29. Cell envelope fractions of cells grown in KB medium supplemented with 100 μM $FeCl_3$ (lane 1), iron-limiting KB medium (lane 2) and iron-limiting KB medium supplemented with 40 μM pseudobactin BN8 (lane 3) were analysed by SDS–PAGE. The positions of the molecular weight standard proteins (left) and the PupB protein (arrow) are indicated.

Table II. Regulation of *pupB*−*lacZ* expression by PupI and PupR

Strains	Genomic mutation	Plasmid	β-Galactosidase activity (U) +Fe	−Fe	ps.BN8
WCS358	−	−	51	168	636
KV51	*pupI*::Tn*5*	−	40	112	156
KV51	*pupI*::Tn*5*	(pMC1*pupI*$^+$*pupR*$^+$)	40	136	1912
KV51	*pupI*::Tn*5*	(pMC2*pupI*$^+$)	91	635	780
BWV29	*pupR*::Ω	−	56	244	274
BWV29	*pupR*::Ω	(pMC1*pupI*$^+$*pupR*$^+$)	36	147	2112
BWV29	*pupR*::Ω	(pMC2*pupI*$^+$)	83	982	1080
WCS358	−	(pMC2*pupI*$^+$)	126	623	2914
WCS358	−	(pMC1*pupI*$^+$*pupR*$^+$)	75	140	2393

All strains harbour plasmid pMW2 carrying a transcriptional *pupB*−*lacZ* fusion. Cells are grown as described in Table I.

inactivated gene into the chromosome (see Materials and methods). The resulting mutant BWV29 exhibited pseudobactin-independent synthesis of the PupB receptor (Figure 4). β-galactosidase activity resulting from expression of the *pupB*−*lacZ* construct in strain BWV29(pMW2) was also constitutive, although at a significantly lower level than in the parental strain (Table II). Introduction of plasmid pMC2 carrying *pupI* into this mutant resulted in an increase in pseudobactin-independent expression, whereas normal regulation was re-established by plasmid pMC1 containing both regulatory genes, *pupI* and *pupR* (Table II). These results supported a function of PupR in repressing PupB synthesis in the absence of pseudobactin BN8 and showed in addition that an intact *pupR* gene is required for optimal *pupB* promoter activity under induction conditions.

Introduction of plasmid pMC2 carrying *pupI* into the wild-type strain WCS358 resulted in very high levels of *pupB* promoter activity even in the absence of pseudobactin BN8, whereas transcription remained normally regulated when *pupI* and *pupR* were both overexpressed (Table II). These results were consistent with the idea that PupI acts as a positive regulator of *pupB* transcription, whereas PupR prevents transcriptional activation by PupI in the absence of pseudobactin BN8. The observation that the amount of chromosomally encoded PupR was not sufficient to repress completely the activity of the plasmid-encoded PupI under iron limitation was suggestive of repression of PupI by PupR through the formation of a stoichiometric complex rather than by enzymatic interaction.

Since many regulatory proteins control the transcription of their own genes, the possibility of autoregulation of *pupI* and *pupR* was examined by measuring β-galactosidase activity in the genomic mutants BWV29 and KV51 harbouring the *pupI*−*lacZ* fusion pMW1. No differences in promoter activity were found between the mutants lacking the regulatory proteins and the parental strain (Table I). Therefore, PupI and PupR synthesis appeared not to be autoregulated.

Function of the PupB receptor in signal transduction

Pseudobactin-dependent activation of the *pupB* promoter was abolished in the chromosomal *pupB* mutant KV53 (Table III). Apparently, the PupB protein is required for transcriptional activation of its structural gene. Since the transport activity of the receptor is dependent on the TonB energy coupling system, it was determined whether the TonB protein

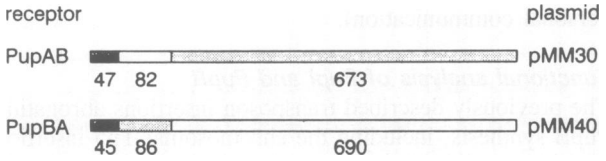

Fig. 5. Schematic representation of the chimeric proteins. The open bar represents PupA, the dotted bar represents PupB, and the signal sequence is indicated by the black bar. The length of the different domains is shown underneath each bar in number of amino acid residues.

is also necessary for induction of *pupB* expression. Therefore, the *pupB*−*lacZ* fusion pMW2 was introduced into the *tonB* mutant TE156 (Bitter *et al.*, 1993). Since strain TE156 is unable to grow under iron limitation, activity of the *pupB* promoter in this mutant was studied in the presence of iron. Under these conditions, the mutant secretes siderophore in high amounts and iron-regulated promoters, which are normally repressed, are transcribed (Bitter *et al.*, 1993). No induction in β-galactosidase activity in response to the presence of pseudobactin BN8 could be observed in TE156(pMW2) (Table III). Thus, PupB and the TonB protein are both essential for siderophore-dependent expression of the *pupB* gene. It is possible that, for induction, the ferric pseudobactin complex has to be transported via the PupB receptor into the periplasm, in order to mediate signal transduction. However, the chromosomal *pupB* mutant KV53 has 50% residual uptake of ferric pseudobactin BN8 (Koster *et al.*, 1993), suggesting that the internalized ferric pseudobactin is not providing the signal for induction. This fact led to the hypothesis that the PupB receptor in concert with the TonB system has a specific role in signal transduction besides its transport function.

A topology model has been proposed for the folding of the PupB receptor in the outer membrane (W.Bitter *et al.*, in preparation). According to this model, the most extensive periplasmic domain of PupB is formed by the N-terminal 70 amino acid residues which are thus probably involved in a putative regulatory function of the receptor. To assess the function of this region, a chimeric receptor was constructed in which the first 86 amino acid residues of mature PupB were replaced with the corresponding part of the PupA protein, the receptor for ferric pseudobactin 358 (Figure 5). Replacement of this region does not involve any cell-surface exposed domains and would therefore not alter

Table III. Involvement of the PupB receptor in regulation of *pupB–lacZ* expression

Strain	Genomic mutation	Plasmid	β-Galactosidase activity (U)			
			+Fe	−Fe	psBN8	ps358
WCS358	–	–	51	168	636	ND
TE156	*tonB*::Tn*5*	–	113	ND	93[a]	ND
KV53	*pupB*::Tn*5*	–	45	85	117	ND
KV53	*pupB*::Tn*5*	(pMM1*pupB*$^+$)	78	135	493	ND
KV53	*pupB*::Tn*5*	(pMM30*pupAB*$^+$)	84	110	150	ND
KV53	*pupB*::Tn*5*	(pMM40*pupBA*$^+$)	96	760	656	ND
WCS3 58	–	(pMM40*pupBA*$^+$)	58	600	ND	ND
JM205	*sid*::Tn*5*	(pMM40*pupBA*$^+$)	55	185	ND	661
KV51	*pupI*::Tn*5*	(pMM40*pupBA*$^+$)	59	142	ND	ND

All strains harbour plasmid pMW2 carrying the *pupB–lacZ* fusion. Cells were grown as described in Table I.
ND, not determined.
[a]For this strain additional iron is added to permit growth of the strain.

the ferric siderophore binding capacity of the protein. A second hybrid receptor was constructed consisting of the signal sequence and the first 86 amino acid residues of PupB fused to the C-terminal 690 amino acid residues of the PupA receptor (Figure 5). The fusion sites in the hybrid receptors are located within the first postulated transmembrane domain which is highly conserved between PupA and PupB. The hybrid genes were made by using newly introduced restriction sites created by PCR-mediated mutagenesis (see Materials and methods). Introduction of the restriction sites resulted in a change of the serine residues at positions 131 and 133 in the PupA and the PupB receptor, respectively, into alanine. The hybrid *pupAB* and *pupBA* genes were cloned in the broad host range vector pML130 behind the *lac* promoter resulting in the plasmids pMM30 and pMM40, respectively (Figure 5).

The chimeric receptors were first tested for their ferric pseudobactin transport capacity. Since strain WCS358 has multiple outer membrane receptors for the same ferric pseudobactin complex, it was not possible to study the transport activity of the chimeric receptors in the *pupA* and *pupB* mutants of this strain. Therefore, the constructs were introduced in *Pseudomonas* sp. A124 which does not possess outer membrane receptors for utilization of pseudobactin BN8 or pseudobactin 358 (Koster *et al.*, 1993). Plasmid pMM30, carrying the *pupAB* gene, provided strain A124 with the ability to utilize specifically pseudobactin BN8 as determined by a bioassay based upon reversal of EDDA-induced iron starvation. A124(pMM30) could grow as efficiently with ferric pseudobactin BN8 as iron source as strain A124 harbouring pMM1 carrying the intact *pupB* gene (data not shown). Thus, replacement of the N-terminal domain had not altered the transport ability and specificity of the PupB receptor. Similarly, it was established that the hybrid PupBA receptor still took up ferric pseudobactin 358.

The chimeric receptors were subsequently tested for restoration of *pupB–lacZ* induction in strain KV53, the *pupB* mutant of strain WCS358. Strain KV53 harbouring pMM1 carrying an intact *pupB* gene exhibited induction of the *pupB* promoter comparable to that of the wild-type strain, whereas in KV53(pMM30) expressing the PupAB hybrid receptor, no promoter activity was observed (Table III). Since the chimeric receptor still transports iron via pseudobactin BN8, this result confirmed a distinct function of PupB in regulation of gene expression. Interestingly, introduction of the *pupBA* gene in strain KV53 resulted in high β-galactosidase activity under iron-depleted conditions independent of the presence of pseudobactin BN8 (Table III). A possible explanation for this result could be that the PupBA receptor mediated activation of the *pupB* promoter in response to its cognate siderophore, pseudobactin 358, which is produced under iron limitation by strain KV53. To investigate this possibility, pMM40 with the hybrid *pupBA* gene was introduced into the wild-type strain WCS358 and a mutant strain JM205, defective in siderophore biosynthesis (Marugg *et al.*, 1985). The presence of the hybrid receptor led to *pupB* promoter activity under iron limitation in the wild-type strain, whereas no *pupB* expression was observed in the biosynthesis mutant unless exogenous siderophore 358 was added to the medium (Table III). This result provided evidence for pseudobactin 358-dependent *pupB* induction mediated by the chimeric PupBA receptor. No *pupB* promoter activity was observed in the *pupI*::Tn*5* mutant KV51(pMM40) which showed that the PupI and PupR proteins are required for this induction. Thus, the chimeric receptor PupBA could induce *pupB* expression via the PupI and PupR proteins in response to the PupA-related siderophore, pseudobactin 358, instead of to pseudobactin BN8. These results demonstrated that the stimulus to which the PupI/PupR system responds is not the ferric siderophore complex itself, but a signal transduced by the receptor in response to the presence of its substrate. Furthermore, the domain of PupB involved in signal transduction is located in the N-terminal 86 amino acid residues of the receptor.

Discussion

The ability of *P.putida* WCS358 to transport iron complexed to a large variety of siderophores seems to be associated with the presence of a large number of outer membrane receptors which are specifically up-regulated when the corresponding siderophore is encountered in the environment (Koster *et al.*, 1993). The expression of the inducible PupB receptor was studied in order to obtain insight in the underlying regulatory network. Two genes, *pupI* and *pupR*, were identified which are involved in controlling pseudobactin BN8-dependent expression of *pupB*. The genes, located immediately upstream of the *pupB* locus, are apparently organized in an

operon. Their expression is strongly increased upon iron deprivation as deduced from experiments with a *pupI–lacZ* transcriptional fusion. The presence of a putative Fur box upstream of the *pupI* gene suggests that the Fur protein of *P.putida* WCS358 is involved in this regulation. Recently, a positive regulatory element, named PfrA, has been identified which is required for expression of siderophore biosynthetic genes under low iron conditions in strain WCS358 (Venturi *et al.*, 1993). This element seems to be specifically involved in iron-responsive regulation of siderophore biosynthesis since activity of the *pupI* promoter was not dependent on the PfrA protein (unpublished results).

The deduced PupI and PupR protein products are predicted to be located in the cytoplasm and inner membrane, respectively. The two proteins display significant homology to the FecI and FecR proteins of *E.coli* (van Hove *et al.*, 1990). No similarity was found with other bacterial regulatory proteins, thus these two systems may comprise a new class of prokaryotic signal transduction systems. Mutational analysis showed that the PupI protein acts as a positive regulator of *pupB* transcription. A *pupI*::Tn*5* mutant no longer exhibited induction of the *pupB* promoter, and complementation of this mutant by the *pupI* gene alone was sufficient to restore *pupB* expression, although in a pseudobactin BN8-independent manner. The fact that a helix–turn–helix motif is predicted in the C-terminal part of the PupI protein is suggestive of a role for PupI in facilitating gene expression by interaction with the *pupB* promoter region. Such a role is supported by the finding that its *E.coli* counterpart FecI binds specifically to the *fecA* operator region (V.Braun, personal communication).

Overexpression of *pupI* resulted in activation of the *pupB* promoter even in the absence of pseudobactin BN8, whereas simultaneous overexpression of *pupR* restored pseudobactin-responsive regulation. Apparently, PupR inhibits the activity of the PupI protein in the absence of the siderophore. Consistent with this role, a mutant deficient in PupR production exhibited PupB synthesis independent of pseudobactin BN8. It should be noted that the *pupB* promoter activity was markedly reduced in this mutant relative to that in the parental strain under induction conditions. This reduction may be due to an effect of the *pupR* mutation on the stability of the *pupI* mRNA or PupI protein. Alternatively, it is possible that the PupR protein also functions as a positive regulator of *pupB* expression by stimulating PupI activity when pseudobactin BN8 is present. Exactly how the PupR protein modulates the activity of PupI is not known. The lack of homology between the PupI/PupR system and other regulatory systems indicates a different mechanism of signal transduction than phosphorylation, which is the most commonly found signalling strategy in bacteria (for a review, see Parkinson, 1993).

In addition to the PupI/PupR system, the PupB receptor was also shown to play a crucial role in signal transduction. Replacement of the N-terminal domain of PupB by the corresponding domain of PupA, the receptor for ferric pseudobactin 358, did not interfere with the transport function of the PupB receptor but did affect its ability to activate *pupB* expression in response to the presence of pseudobactin BN8. This result reveals a specific function of the receptor in regulation of gene expression. Furthermore, a chimeric PupBA protein consisting of the N-terminal 86 amino acid residues of the PupB receptor and

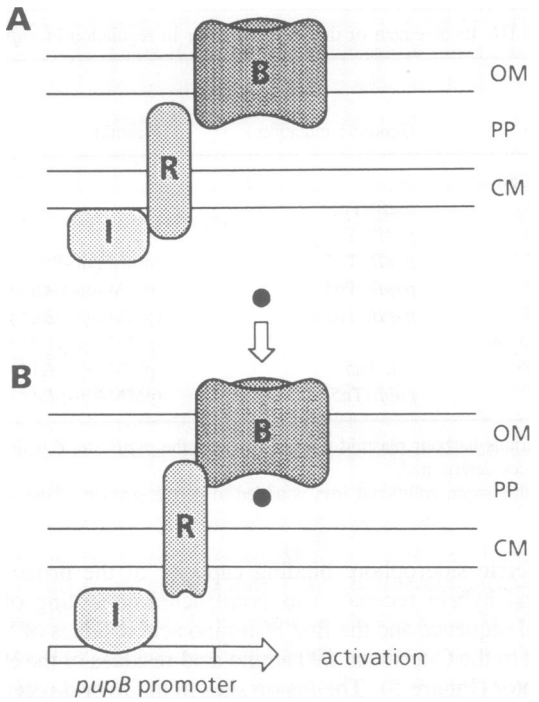

Fig. 6. Model for the signal transduction pathway regulating the expression of the *pupB* gene in response to the presence of pseudobactin BN8. (**A**) In the absence of pseudobactin BN8, the PupR protein (R) represses the activity of the transcriptional activator PupI (I) resulting in down-regulation of *pupB* expression. (**B**) In the presence of pseudobactin BN8 a signal is transduced by the PupB outer membrane receptor (B) to the regulatory system which eliminates the inhibition of PupI activity by PupR resulting in activation of *pupB* transcription. OM, outer membrane; CM, cytoplasmic membrane; PP, periplasm.

the C-terminal 690 amino acid residues of PupA did restore *pupB* induction in the mutant but in response to the PupA-related siderophore, pseudobactin 358, instead of pseudobactin BN8. Therefore, the stimulus to which the two-component system responds is not the ferric siderophore complex itself, but a signal that is transduced by the receptor upon transport of its substrate. The fact that the chimeric receptor composed of only 86 N-terminal amino acid residues of PupB and the remainder of PupA was capable of inducing *pupB* expression in response to pseudobactin 358 shows that the information required for transmitting the signal is located within this N-terminal domain.

An interesting question is how the presence of the substrate triggers the receptor to transmit the signal. It has been proposed that ferric siderophore receptors function as gated channels which are opened upon binding of their substrate through the action of the TonB energy coupling system (Killman *et al.*, 1993; Rutz *et al.*, 1993). Therefore, opening of the channel and the concomitant conformational change of the receptor could be the trigger for signal transduction. The finding that a functional TonB protein is required for activation of *pupB* expression is consistent with this notion.

In conclusion, three proteins, PupB, PupI and PupR, are involved in the siderophore-dependent induction of the *pupB* expression. Additional proteins appear not to be required for the regulation, since PupB is siderophore-dependently regulated when *pupI*, *pupR* and *pupB* are expressed in heterologous *Pseudomonas* strains which do not possess this transport system. Therefore, the following model of siderophore-responsive regulation of *pupB* expression is

Table IV. Bacterial strains, siderophores and plasmids

Strain/siderophore/plasmid	Relevant characteristics[a]	Source/Reference
Strains		
P.putida WCS358	wild-type, Nx^R	Geels and Schippers (1983)
KV51	WCS358*pupI*::Tn*5*, Km^R, Nx^R	Koster *et al.* (1993)
KV53	WCS358*pupB*::Tn*5*, Km^R, Nx^R	Koster *et al.* (1993)
BWV29	WCS358*pupR*::Ω, Sm^R, Nx^R	This work
TE156	WCS358*tonB*::Tn*5*, Km^R, Nx^R	Bitter *et al.* (1993)
JM205	WCS358*sid*::Tn*5*, Km^R, Nx^R	Marugg *et al.* (1985)
Pseudomonas sp. A124	wild-type, Rif^R	Suslow and Schroth (1982)
Pseudomonas sp. BN8	wild-type, Nx^R	Bitter *et al.* (1991)
E.coli PC2495	*recA*, *hsdS*, *lacZY*, *thi*, F′	Phabagen collection
Siderophores		
Pseudobactin BN8	produced by *Pseudomonas* sp. BN8	
Pseudobactin 358	produced by *P.putida* WCS358	
Plasmids	pEMBL18, Ap^R, ColE1 replicon	Dente *et al.* (1983)
pEMBL19	Ap^R, ColE1 replicon	Dente *et al.* (1983)
PRK2013	Km^R, Tra^+, Mob^+, ColE1 replicon	Figurski and Helinski (1979)
pMP220	Tc^R, *'lacZ*	Spaink *et al.* (1987)
pML123	Gm^R, *p*Nm	Labes *et al.* (1990)
pML130	Gm^R, *placZ*	Labes *et al.* (1990)
pJR1	Tc^R, pRK767 carrying *pupI*, *pupR* and *pupB*	Koster *et al.* (1993)
pJRM43	Sm^R, pJRD253 carrying *pupB*	Koster *et al.* (1993)
pUW1	Ap^R, pUC18 carrying *pupA*	W.Bitter, H.Zomer, P.Weisbeek and J.Tommassen (in preparation)
pMW1	Tc^R, pMP220 carrying *pupI*–*lacZ* fusion	This work
pMW2	Tc^R, pMP220 carrying *pupB*–*lacZ* fusion	This work
pMC1	Gm^R, pML123 carrying *pupI*, *pupR*	This work
pMC2	Gm^R, pML123 carrying *pupI*	This work
pMM1	Gm^R, pML130 carrying *pupB*	This work
pMM30	Gm^R, pML130 carrying *pupAB*	This work
pMM40	Gm^R, pML130 carrying *pupBA*	This work
pEW4	Ap^R, pEMBL18 carrying partial *pupR*	This work
pEW5	Ap^R, pEMBL18 carrying partial *pupR*::Ω	This work
pHP45Ω	Ap^R, Sm^R	Prentki and Krisch (1984)

[a]Abbreviations for drug resistance: Nx, nalidixic acid; Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Rif, rifampicin; Sm, streptomycin; Tc, tetracycline.

proposed (Figure 6). Under low iron conditions the regulatory proteins PupI and PupR and small amounts of the PupB receptor are synthesized. In this situation, PupR prevents transcriptional activation of the *pupB* promoter by inhibiting the activity of the PupI protein. When ferric pseudobactin BN8 is present in the environment it will be transported across the outer membrane by the PupB receptor in a TonB-dependent manner. During this transport a signal, probably the conformational change of the receptor, is transduced to the regulatory system. It is attractive to speculate that the PupR protein is the receiver of this signal. After transmission of the signal the PupR protein will no longer repress PupI activity which in turn activates *pupB* gene transcription. Exactly where the PupI and PupR proteins are located in the cell and how the signal is transduced between the different components remains to be determined. According to this model, two functions can be assigned to the PupB receptor, i.e. ferric pseudobactin transport and initiation of the signal transduction pathway that leads to regulation of its own synthesis. This is reminiscent of the situation found for some periplasmic binding protein-dependent transport systems like the phosphate-specific Pst system, which have in addition to their transport function, a role in signal transduction (Cox *et al.*, 1988). The PupB receptor is the first example of an outer membrane protein displaying such a role. FecA, the receptor for ferric dicitrate of *E.coli* was also found to be indispensable for induction of the *fec* genes which has led to the assumption that for induction ferric citrate has to be transported into the periplasm to interact with the regulatory proteins (Zimmermann *et al.*, 1984). Alternatively, the FecA receptor may, in analogy with the PupB system, act as a component of the signal transduction pathway. However, the FecA receptor does not possess an extended periplasmic N-terminal domain like the PupA and the PupB receptor (Pressler *et al.*, 1988). Therefore, it cannot be excluded that the two systems respond to different signals despite the conservation in primary structure of the regulatory proteins.

In strain WCS358 at least three other outer membrane proteins and probably many more are expressed in response to a specific siderophore (Koster *et al.*, 1993). Hence, as many different regulatory systems have to be present to control the synthesis of these proteins. Responding to the transport activity of the receptor instead of to the ferric siderophore in the periplasm could be an efficient means to avoid cross-talk between the different systems.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this work are listed in Table IV. *Pseudomonas* strains were grown at 30°C in King's medium B (KB) (King *et al.*, 1954) or in RSM medium (Buyer *et al.*, 1989) supplemented when required with 100 μM $FeCl_3$ or 40 μM pseudobactin. *E.coli* was cultured at 37°C in LB medium (Miller, 1972). For *Pseudomonas*, antibiotics were used at the following concentrations: nalidixic acid, 25 μg/ml; rifampicin, 40 μg/ml; tetracycline, 40 μg/ml; kanamycin, 50 μg/ml; gentamicin, 50 μg/ml; streptomycin, 50 μg/ml and piperacillin, 75 μg/ml. For *E.coli*, the antibiotics used were tetracycline, 10 μg/ml; kanamycin, 50 μg/ml; gentamicin, 25 μg/ml; streptomycin, 25 μg/ml; and ampicillin, 50 μg/ml.

Plasmids and recombinant DNA techniques

Plasmids used in this study are listed in Table IV. For the construction of pMW1 carrying the *pupI*−*lacZ* transcriptional fusion, the 0.5 kb *StuI*−*PvuII*(1) fragment of plasmid pJR1 (Figure 1) was cloned into pEMBL18, excised with *Eco*RI and *Pst*I and ligated in pMP220 in the proper orientation to direct *lacZ* transcription. Plasmid pMW2, carrying the *pupB*−*lacZ* fusion, was constructed by cloning the 1.2 kb *SalI*(1)−*SalI*(2) fragment of pJR1 (Figure 1) in pEMBL18, followed by ligation into pMP220 in the proper orientation using the *Eco*RI and *Pst*I restriction sites. Plasmid pMC2, carrying the *pupI* gene, was constructed by cloning the 1.0 kb *StuI*−*Sma*I(2) fragment of pJR1 (Figure 1) in pEMBL18, followed by ligation in vector pML123. To obtain construct pMC1, with the *pupI* and *pupR* genes, the 3.5 kb *Eco*RI−*Pst*I fragment of pJR1 (Figure 1) was ligated into pEMBL19, excised with *Bam*HI and *Cla*I and ligated in pML123. The plasmids pMC1 and pMC2 were constructed in such a way that the direction of transcription of the genes was in the opposite orientation with respect to the neomycin promoter. Plasmid pEW4, which was used for the construction of the *pupR* chromosomal mutant, was made by ligation of the 1.5 kb *StuI*−*SalI*(1) fragment of pJR1 (Figure 1) into pEMBL18. Plasmid pMM1, carrying the *pupB* gene, was constructed by cloning the 4 kb *Sma*I(3)−*Sma*I(4) fragment of plasmid pJR1 (Figure 1) into pML130.

Plasmids were isolated by using the rapid procedure described by Birnboim (1983). Digestions with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments and ligation with T4 DNA ligase were performed as described by Maniatis *et al.* (1982). Plasmids were introduced into *E.coli* by transformation using the calcium chloride procedure (Cohen *et al.*, 1972), and into *Pseudomonas* by triparental mating (Marugg *et al.*, 1988).

Determination of nucleotide sequence

DNA segments of the 1.7 kb region of pJR1 upstream of the *pupB* gene (Figure 1) were obtained by digestion with different restriction endonucleases and ligated into the corresponding sites of pEMBL18 and pEMBL19. The constructs were encapsidated as single-stranded DNA after superinfection with phage M13-IR1. Nucleotide sequences were determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using [α-^{35}S]dATP for labelling and 7-deaza-dGTP (Boehringer Mannheim, Germany) instead of dGTP to avoid compression in the sequencing gels. The DNA fragments were separated with a Bio-Rad electrophoresis system.

Cell envelope preparations and SDS−PAGE

Fractions containing outer membranes were isolated by centrifugation of ultrasonically disrupted cells (15 min at 10 000 *g*) followed by extraction with 3% sarkosyl (de Weger *et al.*, 1986). SDS−PAGE was performed on 8% acrylamide gels as described by Laemmli (1970).

Enzyme assay

To determine β-galactosidase enzyme activity cells were grown in RSM medium with the required supplements until late log phase. Enzyme activity of 200 μl cells was determined by using *o*-nitrophenyl-β-galactoside (ONPG) as a substrate as described by Miller (1972). The data are representative of three independent experiments.

Construction of a genomic mutant by gene replacement

For insertional inactivation of *pupR*, plasmid pEW4 was used, a derivative of pEMBL18 carrying a part of the *pupR* gene. The 175 bp *Sma*I fragment located in *pupR* was replaced by the Ω interposon containing a streptomycin resistance gene (Prentki and Krisch, 1984), using plasmid pHP45Ω as the source of the interposon. The resulting construct, pEW5, was introduced into strain WCS358 by electroporation (Hattermann and Stacey, 1990). Since pEW5 cannot replicate in *Pseudomonas*, streptomycin resistance can only be established by homologous recombination of the inactivated *pupR* gene into the chromosome. The streptomycin-resistant colonies were tested for piperacillin sensitivity to confirm the loss of plasmid sequences.

Construction of hybrid receptor genes

The following oligonucleotides were used to introduce a unique restriction site at equivalent positions in the *pupA* and *pupB* sequence: 1, 5′-GGCCAAATCGAGCTAGCAGCGACCA-3′; 2, 5′-TGGTCGCTGCTAGCTCGATTGGCC-3′; 3, 5′-GGCGCCCTGGAGCTAGCCGCGGTGT-3′; 4, 5′-ACACCGCGGCTAGCTCCAGGGCGCC-3′. The oligonucleotides were complementary to either one of the strands of *pupA* (1 and 2) or the *pupB* gene (3 and 4) and contained mismatches (underlined) to introduce a unique restriction site for *Nhe*I. DNA fragments of the *pupB* gene were amplified by PCR using plasmid pJRM43 as a template, the primers 3 and 4 containing the *Nhe*I site and primers complementary to sequences upstream and downstream of the *pupB* gene. In the same way fragments of the *pupA* gene were obtained using plasmid pUW1 as a template. The hybrid genes were constructed by cloning the fragments using the *Nhe*I site in different combinations in plasmid pML130 behind the *lac* promoter (Figure 5).

Siderophore utilization

Pseudobactins were harvested from cultures grown at 30°C for 48 h in RSM medium as described previously (Yang and Leong, 1984). Pseudobactin utilization of *Pseudomonas* strains was determined by reversal of iron starvation induced by ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDA). Bacterial suspensions were added to KB agar with 50 μg/ml EDDA at a concentration of 1000 c.f.u./ml. Filter paper discs were placed on agar containing 4 μl of the different siderophore solutions (100 μM), and after 24 h of incubation at 30°C, the plates were examined for bacterial growth.

Computer analyses

Putative membrane spanning domains were identified using the TOPRED program developed by von Heijne (1992). For comparison of the amino acid sequences of PupI and PupR with proteins present in the SwissProt sequence database, the FASTA program was used (Pearson and Lipman, 1988). Sequences were analysed using programs included in the program package PC/GENE (IntelliGenetics, Inc.).

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Note added in proof

The sequence data of *pupI* and *pupR* have been deposited in the EMBL Data Library under the accession number X77918.